RayBio® Human Acute Kidney Injury Antibody Array 1 (G-Series)

Patent Pending Technology

User Manual (Revised July 2, 2012)

RayBio® Human Acute Kidney Injury Antibody Array G-Series
Cat# AAH-AKI-G1-4

RayBio® Human Acute Kidney Injury Antibody Array G-Series
Cat# AAH-AKI-G1-8

RayBio® Human Cytokine Antibody Array G-Series
Testing Service
Cat# AAH-SERV-G

Please read manual carefully
before starting experiment

RayBiotech, Inc.
the protein array pioneer company

We provide you with excellent Protein Array systems and services

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Website: www.raybiotech.com Email: info@raybiotech.com

RayBiotech, Inc., the Protein Array Pioneer Company, strives to research and develop new products to meet demands of the biomedical community. RayBiotech's patent-pending technology allows detection of up to 507 cytokines, chemokines and other proteins in a single experiment. Our format is simple, sensitive, reliable, reproducible and cost-effective.

Our product offerings include:

1. Protein (antigen) Arrays
2. RayBio® Cytokine Antibody Arrays
   - C Series (Membrane, chemiluminescence detection)
   - G-Series (Glass chip, fluorescence detection)
3. Pathway- and disease-focused antibody arrays
   - Angiogenesis Antibody Arrays
   - Apoptosis Antibody Arrays
   - Atherosclerosis Antibody Arrays
   - Chemokine Antibody Arrays
   - Growth Factor Antibody Arrays
   - Inflammation Antibody Arrays
   - MMP Antibody Arrays
   - Obesity Antibody Arrays
4. Quantibody® Multiplex ELISA Arrays
5. RayBio® L-Series Biotin Label-based Antibody Arrays
6. RayBio® E-Series Competition-based Antibody Arrays
7. RayBio® Phosphorylation Antibody Arrays
   - Receptor Tyrosine Kinases
   - EGFR and ErbB family (site-specific phosphorylation)
8. Over 700 different ELISA kits
9. EIA (Competitive ELISA) kits
10. Cell-based Phosphorylation Assay
11. Over 10,000 different antibodies
12. Recombinant proteins
13. Peptide
14. Recombinant antibodies

Protocol for RayBio® Human Acute Kidney Injury Antibody Array G-Series 1

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RayBio® Cytokine Antibody Arrays are patent-pending technology.
RayBio® is the trademark of RayBiotech, Inc.

I. Introduction

New techniques such as cDNA microarrays have enabled us to analyze global gene expression[1-3]. However, almost all cell functions are executed by proteins, which cannot be studied simply through DNA and RNA techniques. Experimental analysis clearly shows disparity can exist between the relative expression levels of mRNA and their corresponding proteins[4]. Therefore, analysis of the proteomic profile is critical.

RayBiotech, The Protein Array Pioneer Company, introduced the first protein arrays to the market in 2001 and continues to lead in the development of innovative protein array technologies, such as the RayBio Human Acute Kidney Injury Antibody Array.

Acute kidney injury is a common complication among ambulatory and hospitalized patients. It is a rapidly progressive illness that independently predicts excess morbidity and mortality. It is critical to early detect acute kidney injury and distinguish it from prerenal azotemia and chronic kidney disease at the time of patient presentation to rapidly manage associated illness. However, serum creatinine, a standard marker of kidney function, does not distinguish acute kidney injury from prerenal azotemia[5] or chronic kidney disease. In addition, the initial measurement of serum creatinine cannot reflect the extent of injury because its accumulation always lags behind the insult[6].

Concurrently, the potential for improving risk stratification, informing clinical decision making, and guiding pharmaceutical development recently led the American Society of Nephrology to designate the development of novel AKI biomarkers a top research priority[7]. The response over a few years resulted in the identification of nearly 20 potential markers. Some of the more promising of these include urine or plasma Neutrophil Gelatinase-associated Lipocalin (NGAL)[8], Kidney Injury Molecule-1 (KIM-1)[9], Cystatin C[10], Liver Fatty-acid Binding Protein (L-FABP)[11], Monocyte Chemoattractant Protein 1 (MCP-1)[12] and Trefoil Factor 3 (TFF3)[13].

Traditionally, urine proteins or cytokines are detected by using ELISA. However, RayBio® Human Acute Kidney Injury Antibody Array G Series can detect 20 protein biomarkers simultaneously with small amount of sample. It is a great tool in the acute kidney injury research areas including drug toxicity monitoring, kidney transplantation rejection reaction monitoring, and kidney injury early detection.

1. Tang X, Marciano DL, Leeman SE, Amar S. LPS induces the interaction of a transcription factor, LPS-induced TNF-a factor, and STAT6(B) with effects on multiple cytokines. *PNAS*. 2005;102(14): 5132-5137.

2. Xu Y, Kulkosky J, Acheampong E, Nunnari G, Sullivan J, Pomerantz RJ. HIV-1-mediated apoptosis of neuronal cells: Proximal molecular mechanisms of HIV-1-induced encephalopathy. *PNAS*. 2004;101(18): 7070-7075.

3. El-Hage N, Gurwell JA, Singh IN, Knapp PE, Nath A, Hauser KF. Synergistic increases in intracellular Ca(2+), and the release of MCP-1, RANTES, and IL-6 by astrocytes treated with opiates and HIV-1 Tat. *Glia*. 2005 Apr 15;50(2):91-106.

4. Oh HS, Moharita A, Potian JG, Whitehead IP, et al. Bone Marrow Stroma Influences Transforming Growth Factor-β Production in Breast Cancer Cells to Regulate c-myc Activation of the Preprotachykinin-I Gene in Breast Cancer Cells. *Cancer Res*. 2004;64: 6327-6336.

5. Bonventre JV, Weinberg JM. Recent advances in the pathophysiology of ischemic acute renal failure. *J Am Soc Nephrol.* 2003;14:2199–210.

6. Lameire N, Hoste E. Reflections on the definition, classification, and diagnostic evaluation of acute renal failure [Editorial]. *Curr Opin Crit Care*. 2004;10:468–75.

7. American Society of Nephrology Renal Research Report. *J Am Soc Nephrol*. 16: 1886–1903, 2005.

8. Mishra J, Dent C, Tarabishi R, Mitsnefes MM, Ma Q, Kelly C, Ruff SM, Zahedi K, Shao M, Bean J, Mori K, Barasch J, Devarajan P: Neutrophil gelatinase-associated Lipocalin (NGAL) as a biomarker for acute renal injury after cardiac surgery. *Lancet* . 2005;365: 1231–1238.

9. Han WK, Bailly V, Abichandani R, Thadhani R, Bonventre JV: Kidney Injury Molecule-1(KIM-1): A novel biomarker for human renal proximal tubule injury. *Kidney Intl*. 62: 237–244, 2002.

10. Herget-Rosenthal S, Marggraf G, Husing J, Goring F, Pietruck F, Janssen O, Philipp T, Kribben A: Early detection of acute renal failure by serum cystatin C. *Kidney Intl*. 66: 1115–1122, 2004.

11. Yamamoto T, Noiri E, Ono Y, Doi K, Negishi K, Kamijo A, Kimura K, Fujita T, Kinukawa T, Taniguchi H, Nakamura K, Goto M, Shinozaki N, Ohshima S, Sugaya T: Renal L-type fatty acid–binding protein in acute ischemic injury. *J Am Soc Nephrol*.18: 2894–2902, 2007.

12. Munshi R, Johnson A, Siew ED, Ikizler TA, Ware LB, Wurfel MM, Himmelfarb J, Zager RA. MCP-1 gene activation marks acute kidney injury. *J Am Soc Nephrol*. 2011 Jan;22(1):165-75.

13. Yu Y, Jin H, Holder D, Ozer JS, Villarreal S, Shughrue P, Shi S, Figueroa DJ, Clouse H, Su M, Muniappa N, Troth SP, Bailey W, Seng J, Aslamkhan AG, Thudium D, Sistare FD, Gerhold DL. Urinary biomarkers trefoil factor 3 and albumin enable early detection of kidney tubular injury. *Nat Biotechnol*. 2010 May; 28(5):470-7.

II. Product Information

A. Storage Recommendations:

For best results, we recommend storing the entire kit at –20°C or –80°C upon arrival and using the kit within 6 months of receipt. RayBiotech warranties this product for 6 months if stored in this manner.

Once thawed, store glass chips and 1X Blocking Buffer at –20°C or –80°C and all other component at 4°C. After thawing, the entire kit should be used within 3 months. RayBio® Antibody Array kits are robust and will retain full activity even if accidentally stored at room temperature (RT) for up to 24 hours.

B. Materials Provided

Item	Description	AAH-CYT-G8-4	AAH-CYT-G8-8
AAH-AKI-G1	RayBio® Human Acute Kidney Injury Microarray Glass Chip*	1 chip with 4 Sub-arrays*	1 chip with 8 Sub-arrays*
0103002 – HAKI1	Biotin-Conjugated Anti-Cytokines	1 ea	2 ea
0103004-H	1,500X HiLyte Plus™ 532 Streptavidin-Fluor†	1 ea	1 ea
0103004-B	1X Blocking Buffer	10 ml	20 ml
0103004-W‡	20X Wash Buffer I ‡	30 ml	30 ml
0103004-W‡	20X Wash Buffer II ‡	30 ml	30 ml
0103004-L	2X Cell Lysis Buffer (optional)	10 ml	10 ml
Other Kit Components: Manual, Adhesive Plastic Strips, 30 ml Centrifuge Tube			

* Kit contains 1 pre-assembled glass chip with either 4 or 8 printed sub-arrays per chip (in sealed plastic envelope)
[*NOTE: In some cases, 2 chips x 4 sub-arrays/chip may be substituted in kits containing 8 sub-arrays*]

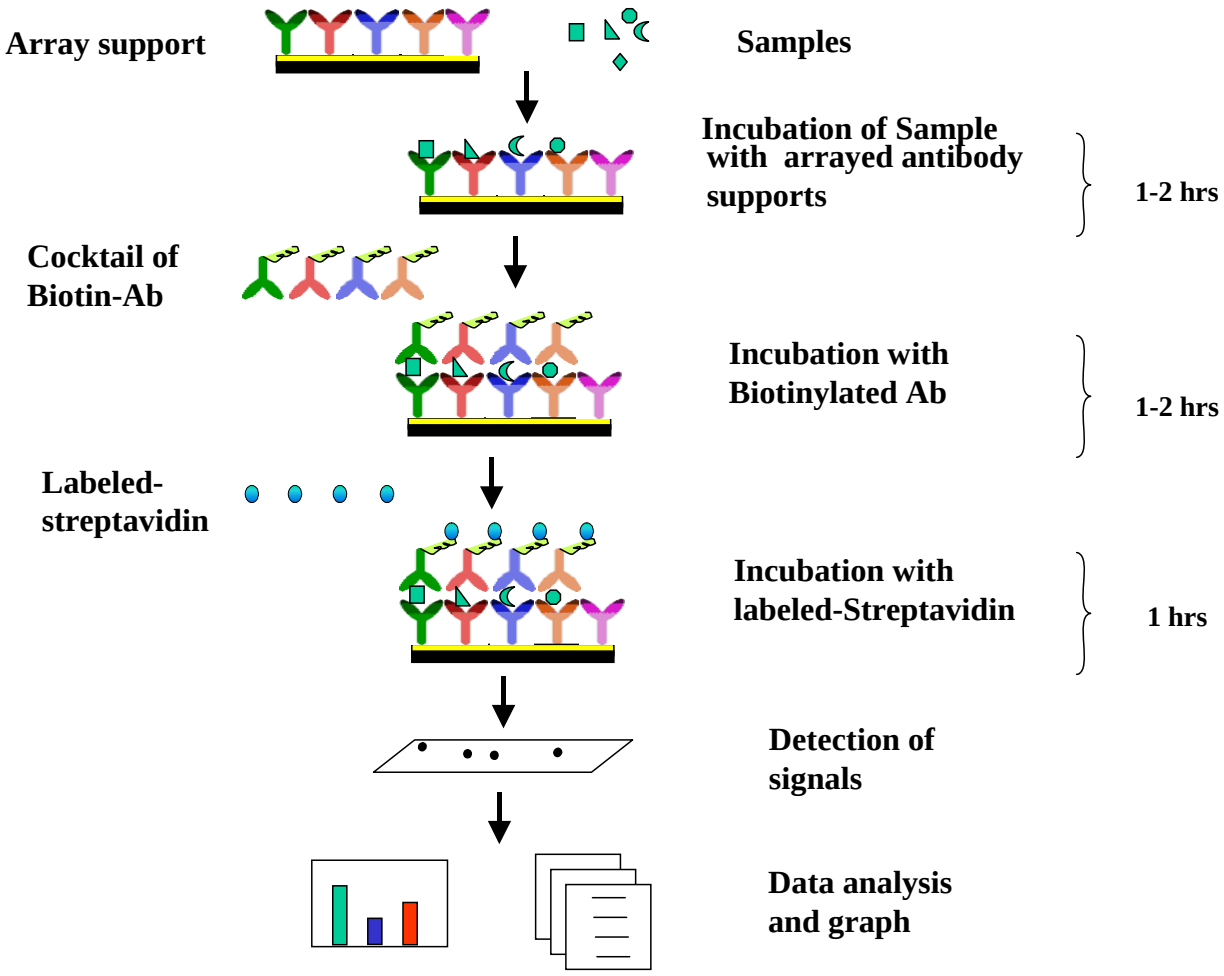
† This fluor is patent-pending technology from Anaspec, Inc.

‡ Wash Buffers are sold as sets

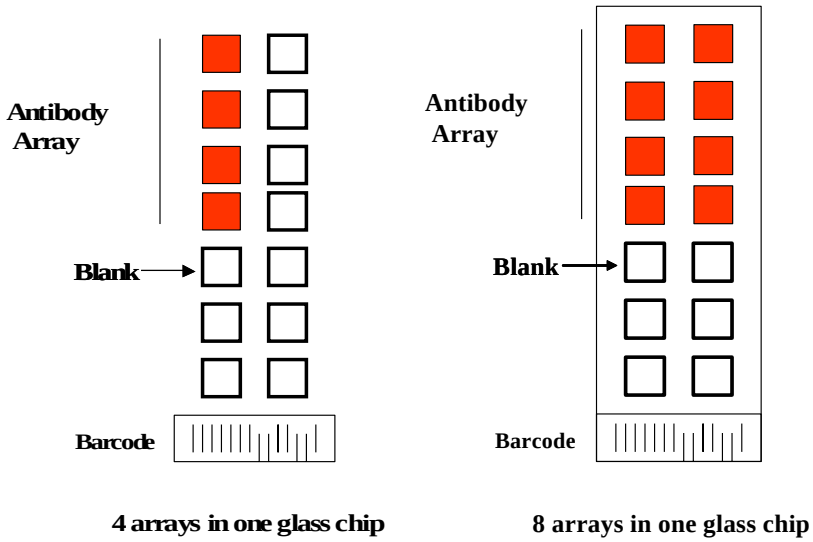
C. Additional Materials Required

- Small plastic boxes or containers
- Pipettors, pipette tips and other common lab consumables
- Orbital shaker or oscillating rocker
- Aluminum foil
- Gene microarray scanner or similar laser fluorescence scanner (see pages 9 & 15)

D. How It Works



E. RayBio® G Series Glass Chip Layout



III. Helpful Tips and General Considerations

A. Preparation and Storage of Samples

1. General Considerations:
   - Freeze samples as soon as possible after collection.
   - Avoid multiple freeze-thaw cycles. If possible, sub-aliquot your samples prior to initial storage.
   - Spin samples hard (5-10 minutes at 10K to 15K RPM) immediately prior to incubation of samples with array.
   - Optimal sample concentrations may need to be determined empirically based on the signal intensities of spots and background signals obtained.
   - Most samples will not need to be concentrated. If concentration is required, we recommend using a spin-column concentrator with a chilled centrifuge.
2. Recommended Sample Volumes and Dilution Factors

NOTE: All sample dilutions should be made using 1X Blocking Buffer. For all sample types, final sample volume = 50-100 µl per sub-array

- Cell Cultured Media: Neat (no dilution needed)
- Serum & Plasma: 5-fold to 10-fold dilution
- Most other Body Fluids: Neat or 2-fold to 5-fold dilution
- Cell and Tissue Lysates: Minimum 5-fold to 10 fold to equal concentrations of total protein in each lysate sample.
  - You must determine the total protein concentration of each lysate/homogenate. We recommend using the BCA method (available from Pierce); it is insensitive to detergents commonly found in lysis buffers.
  - Minimum Recommended Total Lysate Protein Concentration (prior to sample dilution) = 5 µg/µl
  - Minimum Recommended Dilution of Lysates (prior to sample incubation): 5-fold to 10 fold with 1X Blocking Buffer. Dilute all lysate samples to the same final concentration of total lysate protein in 1X Blocking Buffer to 50 µl to 100 µl final volume.

- To start, we recommend using 100-200 µg of total protein in 1 ml of 1X Blocking Buffer (final volume) per sub-array.
- Optimal amounts of total lysate protein may range from 50-1000 µg per sub-array. Based upon background and spots intensities, you may increase or decrease the amount of protein used in subsequent experiments.

- **Other Liquid Sample Types**: Most often Neat or 2-fold to 5-fold. However, optimal dilutions should be determined empirically.

3. Sample Preparation

For tips on sample preparation, please visit our Website:
http://www.raybiotech.com/Tech-Support/SampleTips.pdf

B. Handling Glass Chips

- Do not remove glass chip from assembly until Step 16.
- Hold the slides by edges only; do not touch the surface.
- Handle all buffers and slides with powder-free gloves.
- Dry glass chip completely before proceeding to Step 3.
- Handle and dry glass chip in clean environment.
- Avoid breaking glass chip when removing the chamber assembly.

C. Incubations and Washes

- Cover incubation chamber with adhesive film (included in kit) to prevent evaporation, particularly during incubation or wash steps >2 h or with liquid volumes <100 µl per well.
- Perform all incubation and wash steps under gentle rotation or rocking motion (~0.5 to 1 cycle/s).
- Wash steps in Wash Buffer II and all incubation steps may be performed overnight at 4°C.
  - Overnight sample incubations are the most effective at increasing sample spot intensities.
- Avoid cross-contamination of samples to neighboring wells

- To remove Wash Buffers and other reagents from chamber wells, you may invert the Glass Chip Assembly to decant, and aspirate the remaining liquid.
- In Wash Steps 6, 12 and 15, you may gently flush wells several times using a wash bottle filled with Wash Buffer I.

D. Scanning and Data Extraction Tips:

For tips on scanning and data extraction, please visit our Website: http://www.raybiotech.com/Tech-Support/ScanningTips.pdf

For a list of recommended scanners, please visit our Website: http://www.raybiotech.com/resources.asp.

See also page 18 of this manual.

IV. Protocol

A. Preparation and Storage of Reagents

NOTE: During this protocol, prepare reagents immediately prior to use and keep working dilutions of all reagents on ice at all times.

1. Blocking Buffer is supplied at 1X concentration. No dilution is required.

2. Wash Buffers I and II are supplied at 20X concentration.
   a). For each glass chip (4 or 8 sub-arrays/chip), dilute 6 ml of 20X concentrate with deionized H_20 to a final volume of 120 ml each of Wash Buffer I & Wash Buffer II.
   b). Wash buffer reagents at working dilution (1X) can be stored at 4°C for up to 1 month. Stock solutions at 20X can be stored 4°C for up to 3 months.

3. Biotin-conjugated Anti-Cytokines are supplied at high concentration in a small liquid bead (typically ~2-5 µl).

a). Spin down the tube prior to reconstitution, as the concentrated liquid bead may have moved to the top of the tube during handling.
b). Prepare stock reagent by adding 300 µl 1X Blocking Buffer to Biotin-Conjugated Anti-Cytokines. Mix well.
c). 1X Biotin-Conjugated Anti-Cytokines may be stored for 2-3 days at 4°C.

4. Streptavidin-Fluor is supplied at 1500x concentration.
a). Mix the tube containing 1500X Streptavidin-Fluor well before use, as precipitants may form during storage.
b). Add 100 µl of 1X Blocking Buffer to tube containing 1500X Streptavidin-Fluor. Mix well.
c). Quantitatively transfer all of Streptavidin-Fluor reagent from the original tube to a larger one, and dilute with 1X Blocking Buffer to a final volume of 1500 µl (ie, 1.5 ml).
d). Wrap tube containing Streptavidin-Fluor with aluminum foil.
e). This working dilution can be stored for 3-5 days at 4°C.

B. Blocking and Incubations

NOTE: Please carefully read Section III of this manual before proceeding

NOTE: Prepare all reagents immediately prior to use as described above (Section IV.A) and before proceeding.

1) Remove the package containing the glass chip assembly from the freezer. Place unopened package on the benchtop and allow the glass chip assembly to equilibrate to room temperature (RT), approx. 15 min. Open package, remove the glass chip assembly and place in laminar flow hood to dry for 1-2 hours.

NOTE: Be sure glass chip is completely dry before proceeding.

2) If necessary, assemble the glass chip into incubation chamber and frame as shown on page 12. (Note: if you slide is already assembled, you can proceed directly to Step 3).

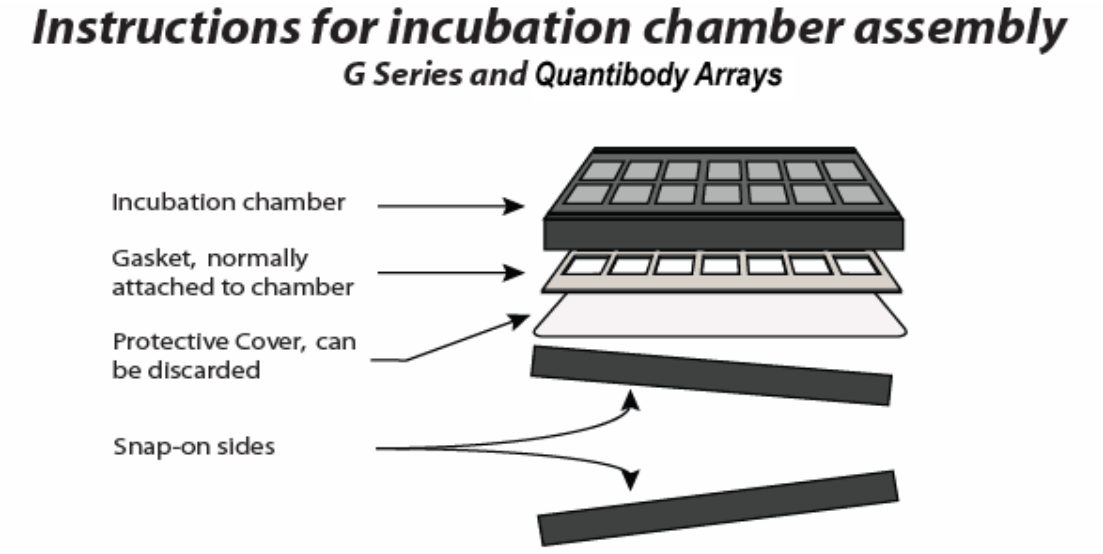
3) Add 100 µl 1 X Blocking Buffer into each well and incubate at RT for 30 min to block slides.

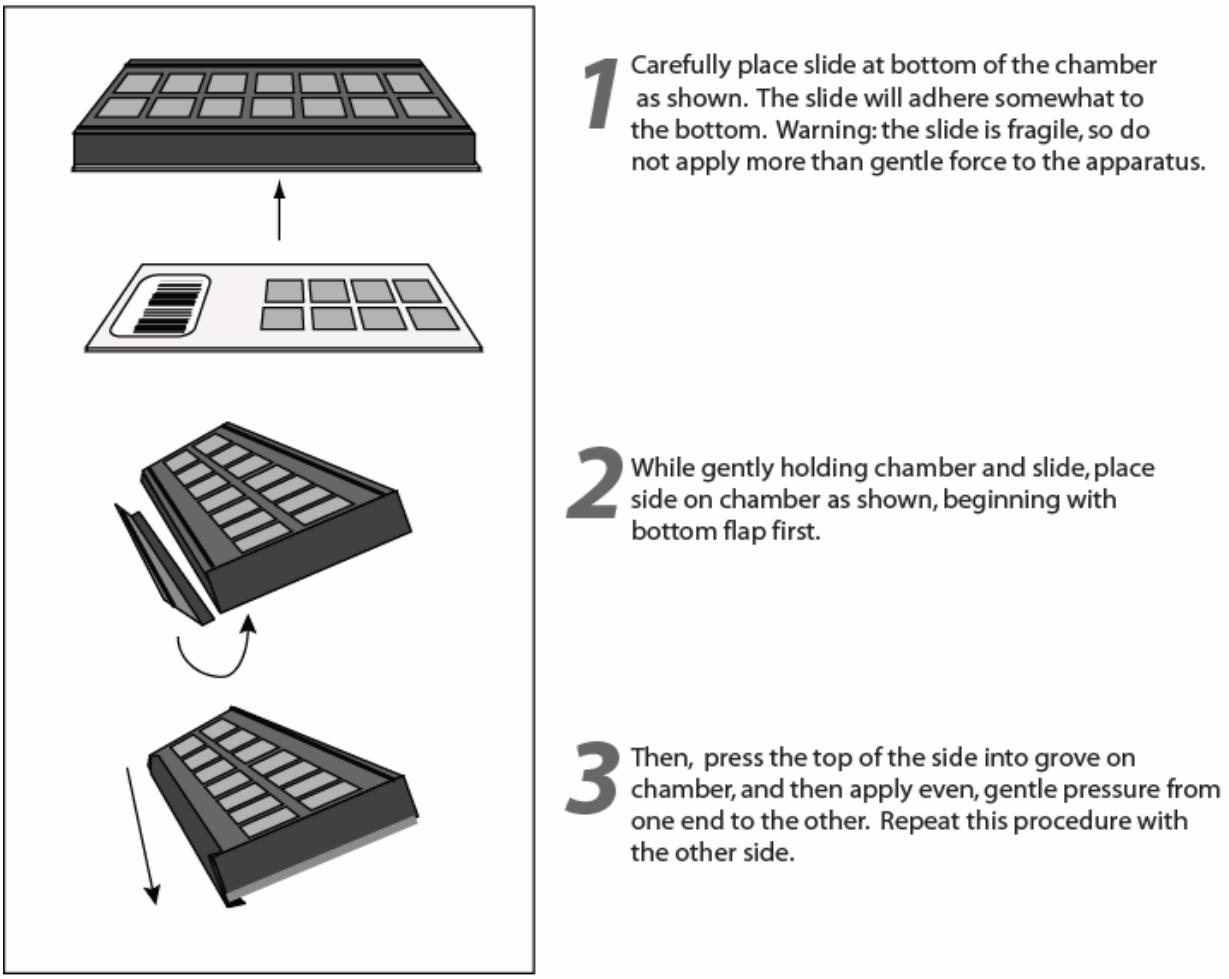
NOTE: Only add reagents or samples to wells printed with antibodies (see diagram on page 5)

4) Decant Blocking Buffer; then aspirate remaining liquid from each well.

NOTE: To aspirate liquid samples or reagents from wells, gently place the pipette tip only in the corners of the well. Do not scrape the pipette tip across the surface of the chip.

5) Add 50 to 100 µl of each sample to each sub-array. Cover the incubation chamber with Adhesive film (included in kit). Incubate arrays with sample at RT for 2 hours. Dilute sample using 1X Blocking Buffer if necessary.


Instructions for incubation chamber assembly
G Series and Quantibody Arrays
Incubation chamber
Gasket, normally attached to chamber
Protective Cover, can be discarded
Snap-on sides


1 Carefully place slide at bottom of the chamber as shown. The slide will adhere somewhat to the bottom. Warning: the slide is fragile, so do not apply more than gentle force to the apparatus.
2 While gently holding chamber and slide, place side on chamber as shown, beginning with bottom flap first.
3 Then, press the top of the side into grove on chamber, and then apply even, gentle pressure from one end to the other. Repeat this procedure with the other side.

6) Remove adhesive film, and carefully aspirate samples from sub-arrays, touching only the corners with your pipette tip.

NOTE: Try to prevent solution from flowing into neighboring wells.

7) Wash 3 x 2 min with 150 µl Wash Buffer I at RT. Be sure to completely remove sample and Wash Buffer each time and use fresh buffer for each wash. Decant final wash solution before proceeding to next step.

8) Obtain a clean container (eg, pipette tip box or slide staining jar) and place glass chip assembly into the container. Add enough 1X Wash Buffer I to submerge the entire glass chip with frame intact (approx. 30-50 ml) and remove all bubbles in wells. Wash 10 min at RT with gentle rocking or shaking.

9) Remove glass chip assembly and invert it to decant liquid. Decant buffer from container and replenish with 1X Wash Buffer I. Submerge the entire glass chip assembly and wash 10 min at RT with gentle rocking or shaking.

10) Remove glass chip assembly and invert to decant liquid. Decant buffer from container and repeat Steps 6 & 7 with Wash Buffer II.

11) Remove glass chip assembly from container and invert it to decant liquid, then carefully aspirate wash buffer from wells, touching only the corners with your pipette tip.

12) Add 70 µl of 1X Biotin-conjugated Anti-Cytokines to each sub-array. Cover incubation chamber with Adhesive film (included in kit). Incubate at RT for 2 hours with gentle rocking or shaking.

13) Carefully aspirate all of the Biotin-conjugated Anti-Cytokine reagent. Wash as described in Step 5 above, first with Wash Buffer I then with Wash Buffer II, making sure to completely remove buffer between washes and after final wash.

14) Add 70 µl of 1X Streptavidin-Fluor to each sub-array. Cover the incubation chamber with Adhesive film (included in kit), then cover entire assembly with aluminum foil to avoid exposure to light or incubate in dark room. Incubate at RT for 2 hours with gentle rocking or shaking.

15) Remove aluminum foil and adhesive film. Carefully aspirate Biotin-conjugated Anti-Cytokine reagent. Wash as described in Step 5 above, first with Wash Buffer I then with Wash Buffer II, making sure to completely remove buffer between washes and after final wash.

16) Remove the glass chip from the frame assembly. Place the whole chip in 30 ml centrifuge tube provided, or slide staining

jar. Add enough Wash Buffer I to cover the whole slide (about 20 ml) and gently rock or shake at RT for 10 min.

17) Decant buffer and repeat wash as described in Step 14 (1 x 10 min with Wash Buffer I). Decant buffer, remove the glass chip from the tube, then gently rinse the slide with de-ionized H_2O using a plastic wash bottle.

18) Remove excess liquid from 30 ml centrifuge tube (provided in kit) and place glass chip into the tube. Centrifuge at 1,000 rpm for 3 minutes to remove water droplets.

C. Obtaining Fluorescent Signal Intensities:

19) Remove slide from tube and allow glass chip to dry in a laminar flow hood for at least 20 minutes. Place chip under an aluminum foil tent to protect it from light. Make sure the slides are absolutely dry before scanning or storage.

20) You may proceed immediately to scanning (Step 21), or you may scan at a later time. You may store the slides at RT indefinitely, provided they are protected from strong light.

Note: Unlike most Cy3 fluors, the HiLyte Plus™ Fluor 532 used in this kit is very stable at RT and resistant to photobleaching on completed glass chips. However, please protect glass chips from strong light and temperatures above RT.

21) Scan the glass chip with a laser scanner (such as Innopsys' InnoScan®) using cy3 or "green" channel (excitation frequency = 532 nm). For tips on scanning, visit our Website: http://www.raybiotech.com/Tech-Support/ScanningTips.pdf

NOTE: If you do not have a laser scanner, for a nominal fee you can send your slide to us for scanning and data extraction using Innopsys' InnoScan, and we will return the results to you. Using using alternate protocols, RayBio® G-Series arrays are also compatible with Li-Cor's Odyssey and other microarray scanners.

V. Interpretation of Results:

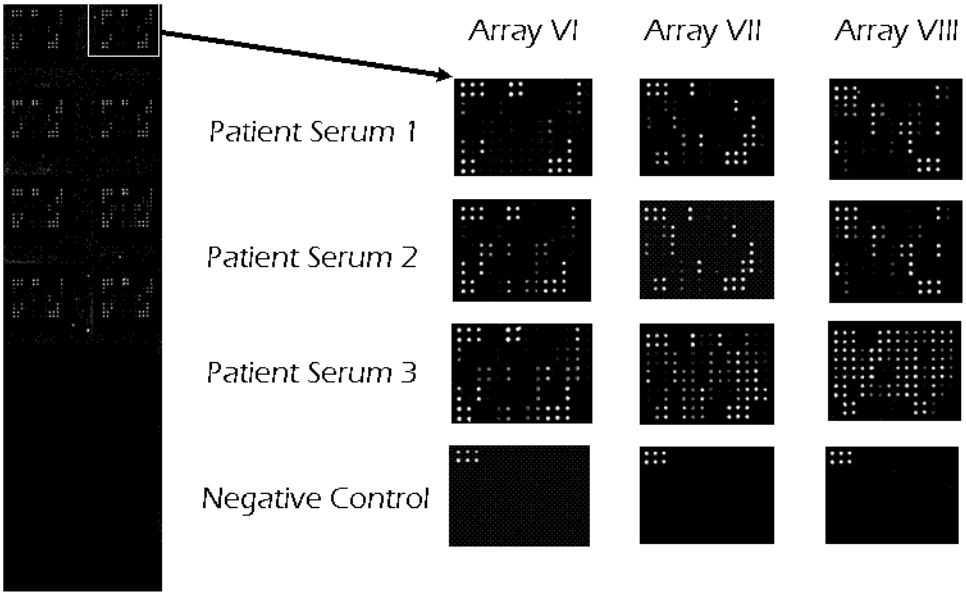
A. Explanation of Controls Spots

Positive Controls (POS1, POS2, POS3) are equal amounts of biotinylated IgGs printed directly onto the array. All other variables being equal, the Positive Control intensities will be the same for each sub-array This allows for normalization based upon the relative fluorescence signal responses to a known control, much as “housekeeping” genes or proteins are used to normalize results in PCR or Western blots, respectively.

Negative Control (NEG) spots are a protein-containing buffer (used to dilute antibodies printed on the array). Their signal intensities represent non-specific binding of Biotin-conjugated anti-Cytokines and/or Streptavidin-Fluor. Negative control signal intensities are usually very close to background signals in each sub-array.

B. Typical results from RayBio® G-Series Antibody Arrays

The following figure shows typical results obtained using RayBio® Antibody Array G-Series Arrays. The images were captured using a GenePix 4000B scanner.



In this example, sera from several patients were incubated with Human Cytokine Arrays 6, 7 & 8, (sold together as Human Cytokine Array G-Series 2000, AAH-CYT-G2000-4 or AAH-CTY-G2000-8) and processed using this standard protocol.

The 6 strong signals of the Positive Control spots in the upper-left corner are useful for proper orientation of the array image.

If scanned using optimal scan settings, 3 distinct Positive Control signal intensities will be seen: POS1>POS2>POS3. If all of these signals are of similar intensity, try increasing or decreasing laser power and/or signal gain settings.

Once you have obtained fluorescence intensity data, you should subtract the background and normalize to the Positive Control signals before proceeding to analysis.

C. Background Subtraction:

Most laser fluorescence scanner software have an option to automatically measure the local background around each spot. As with spot signal intensities, we recommend using MEDIAN background signals. If your resulting fluorescence signal

intensity reports do not include these values (eg, a column labeled as “MED532-B532”), you may need to subtract the background manually or change the default settings on your scanner’s data report menu.

D. Normalization of Array Data:

To normalize signal intensity data, one sub-array is defined as "reference" to which the other arrays are normalized. This choice can be arbitrary. For example, in our Analysis Tool Software, the array represented by data entered in the left-most column each worksheet is the default “reference array.”

You can calculate the normalized values as follows:

$$X(Ny) = X(y) * P1/P(y)$$

Where:

P1 = mean signal intensity of POS spots on reference array
P(y) = mean signal intensity of POS spots on Array "y"
X(y) = mean signal intensity for spot "X" on Array "y"
X(Ny)= normalized signal intensity for spot "X" on Array "y"

The RayBio® Analysis Tool software is available for use with data obtained using RayBio® G-Series Arrays. You can copy and paste your signal intensity data (with and without background) into the Analysis Tool, and it will automatically normalize signal intensities to the Positive Controls.

To order the Analysis Tool, please contact us at +1-770-729-2992 or info@raybiotech.com for more information.

E. Threshold of significant difference in expression:

After subtracting background signals and normalization to Positive controls, comparison of signal intensities for antigen-specific antibody spots between and among array images can be

used to determine relative differences in expression levels of each analyte (ie, protein detected) between samples or groups.

Any ≥1.5-fold increase or ≤0.65-fold decrease in signal intensity for a single analyte between samples or groups may be considered a measurable and significant difference in expression, provided that both sets of signals are well above background (Mean background + 2 standard deviations, accuracy ≈ 95%).

NOTE: In the absence of an external standard curve for each analyte, there is no means of assessing absolute or relative concentrations of different analytes in the same sample using immunoassays. If you wish to obtain quantitative data (ie, concentrations of the various analytes in your samples), try using our Quantibody® Multiplex ELISA arrays instead.

Data Extraction Tips:

- Ignore any comet tails
- Define the area for signal capture for all spots as 110-120 micron diameter, using the same area for every spot.
- Use median signal value, not the total or the mean
- Use local background correction (also median value).
- Exclude obvious outlier data in its calculations.
- Scan all slides at same PMT

VI. RayBio® Human Acute Kidney Injury Antibody Array G Series Map:

Detects 20 cytokines in one experiment

	A	B	C	D	E	F	G	H
1	POS1	POS2	POS3	NEG	KIM-1	ALB	OPN	TFF3
2	POS1	POS2	POS3	NEG	KIM-1	ALB	OPN	TFF3
3	B2M	Clusterin	CXCL16	GPNMB	L-FABP	MCP-1	sTNFRI	Calbindin-1
4	B2M	Clusterin	CXCL16	GPNMB	L-FABP	MCP-1	sTNFRI	Calbindin-1
5	IP-10	Cystatin C	HGF	MIF	NGAL	TIMP-1	VCAM-1	VEGF
6	IP-10	Cystatin C	HGF	MIF	NGAL	TIMP-1	VCAM-1	VEGF
7	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
8	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG

Abbreviations:

POS = Positive Control, NEG = Negative Control, KIM-1 = Kidney Injury Molecule-1, ALB = Albumin, B2M = β2-Microglobulin, OPN = Osteopontin, L-FABP = Liver Fatty-Acid Binding Protein, HGF = Hepatocyte Growth Factor, MIF = Macrophage migration Inhibitory Factor, NGAL= Neutrophil Gelatinase-Associated Lipocalin (Lipocalin 2). All others use standard abbreviations.

VII. Troubleshooting guide

Problem	Cause	Recommendation
No signal for any spots, including Positive Controls	Global detection failure	Adjust scanner settings or re-assemble chip into holder, wash slide 2 x 5 min with 150 µl Wash Buffer II and repeat Steps 12-21.
Similar signal intensities for POS1/2/3	Improper laser power and/or PMT setting	Repeat scan using higher and/or lower laser power or PMT settings
High background signals	Incomplete washes	Carefully follow wash protocols, and/or increase wash times
	Sample concentration is too high	Repeat using lower sample concentration
	Fluor and/or Anti-Cytokines are too concentrated	Review protocol for dilution of reagents
Uneven background and/or missing spots	Bubbles present on chip during incubations	Be sure to completely remove all bubbles from chip surface
	Evaporation during incubation steps	Cover chamber assembly during washes and incubations
	Pooling/precipitation of sample or reagent; Incomplete washes.	Cover chamber assembly and use a rocker or shaker during washes and incubations; carefully follow wash protocols.
	Sample is too concentrated	Repeat experiment using more dilute sample
Randomly scattered high-intensity spots	Dust or other particulates	Dry slides in laminar flow hood and/or use clean containers and powder-free gloves.

Weak or no signals antigen-specific pots + Low Background	Sample is too dilute	Repeat experiment using higher sample concentration
	Improper dilution of Anti-Cytokines or Streptavidin-Fluor	Re-assemble chip into holder, wash 2 x 5 min with 150 µl Wash Buffer II and repeat Steps 12-21. Spin down reagents before diluting and mix well.
	Other Tips	Rescan at higher laser power or signal gain setting
		Repeat using higher sample concentration and/or incubate wi sample O/N at 4°C
		Increase concentration of and/or length of incubation with Biotin-conjugated Anti-Cytokine (+ add'l large volume wash following Biotin-Ab incubation
		Review proper storage conditions for kit components

III. Selected References Citing RayBio® Human G-Series Arrays

1. Mamlouk O, Balagurumoorthy P, Wang K, Adelstein SJ, Kassis AI. Bystander effect in tumor cells produced by iodine-125 labeled human lymphocytes. *Intl J Radiation Biol*. 2012. DOI:10.3109/09553002.2012.702297.

2. Kocaoemer A, Kern S, Kluter H, Bieback K. Human AB serum and thrombin-activated platelet-rich plasma are suitable alternatives to fetal calf serum for the expansion of mesenchymal stem cells from adipose tissue. *Stem Cells*. 2007; 25: 1270-1278

3. Ye Z, Lich JD, Moore CB, Duncan JA, Williams KL, Ting JP-Y. ATP Binding by Monarch-1/NLRP12 is critical for its inhibitory function. *Mol Cell Biol*. 2008;28:1841-1850..

4. Sommer G, Kralisch S, Stangl V, Vietzke A, et al. Secretory products from human adipocytes stimulate proinflammatory cytokine secretion from human endothelial cells. *J Cell Biochem*. 2009;106(4): 729–737.

5. Bouazza B, Kratassiouk G, Gjata B, Perie S, et al. Analysis of growth factor expression in affected and unaffected muscles of oculo-pharyngeal muscular dystrophy (OPMD) patients: A pilot study. *Neuromusc Disorders*. 2009;19(3): 199-206.

6. Dumortier J, Streblow DN, Moses AV, Jacobs JM, et al. Human Cytomegalovirus Secretome Contains Factors That Induce Angiogenesis and Wound Healing. *J Virol*. 2008; 82(13): 6524-655.

7. Keren Z, Braun-Moscovici Y, Markovits D, Rozin A, Nahir M, et al. Depletion of B lymphocytes in rheumatoid arthritis patients modifies IL-8-anti-IL-8 autoantibody network. *Clin Immunol*. 2009. doi:10.1016/j.clim.2009.07.001

8. Rovin BH, Song H, Hebert LA, Nadasdy T, et al. Plasma, urine, and renal expression of adiponectin in human systemic lupus erythematosus. *Kidney Int*. 2005;68: 1825-1833.

9. Duncan JA, Gao X, Huang MT-H, O'Connor BP, Thomas CE, et al. *Neisseria gonorrhoeae* Activates the Proteinase Cathepsin B to Mediate the Signaling Activities of the NLRP3 and ASC-Containing Inflammasome. *J Immunol*. 2009;182: 6460-6469.

10. Pukstadad BS, Ryana L, Floa TH, Stenvika J, et al. Non-healing is associated with persistent stimulation of the innate immune response in chronic venous leg ulcers. *J Dermatol Sc*i. 2009;59(2): 115-122.

11. Park JE, Tan HS, Datta A, Lai RC, et al. Hypoxic Tumor Cell Modulates Its Microenvironment to Enhance Angiogenic and Metastatic Potential by Secretion of Proteins and Exosomes. Mol *Cell Proteom*. 2010;9: 1085-1099.

12. Streblow DN, Dumortier J, Moses AV, Orloff SL, Nelson JA. Mechanisms of Cytomegalovirus-Accelerated Vascular Disease: Induction of Paracrine Factors That Promote Angiogenesis and Wound Healing. Shenk TE, Stinski MF, eds. *Current Topics in Microbiology and Immunology: Human Cytomegalovirus*. Berlin, Heidelberg, Germany: Springer. 2008;325: 397-415.

13. Nolting T, Lindecke A, Koutsilie E, Maschke M, et al. Measurement of soluble inflammatory mediators in cerebrospinal fluid of human immunodeficiency virus-positive patients at distinct stages of infection by solid-phase protein array. *J Neruovirol*. 2009;15(5-6): 390-400.

14. Pannebaker C, Chandler HL, Nichols JJ. Tear proteomics in keratoconus. *Mol Vision*. 2010;16: 1949-1957.

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4-1BB
ACE-2
Acrp30
Activin A
Adiposin
Adipsin
AgRP
ALCAM
α-Fetoprotein
Amphiregulin
Angiogenin
Angiopoietin-1
Angiopoietin-2
Angiostatin
ANGPTL4
Axl
B7-1
BCAM
BCMA
BDNF
β2M
β IG-H3
bFGF
BLC
BMP-4
BMP-5
BMP-6
BMP-7
β-NGF
BTC
CA125
CA15-3
CA19-9
CA IX
Cardiotrophin-1
Cathepsin S
CCL14a
CCL21
CCL-28
CD14
CD23
CD30
CD40
CD40 Ligand
CD80
CEA
CEACAM-1
CK b 8-1
CNTF
Cripto
CRP
CTACK
CXCL16
DAN
Decorin
Dkk-1
Dkk-3
Dkk-4
DPPIV
DR6
Dtk
E-Cadherin
EDA-A2
EGF
EGFR
EG-VEGF
ENA-78
Endoglin
Eotaxin
Eotaxin-2
Eotaxin-3
Ep CAM
ErbB2
ErbB3
EPO R
E-Selectin
Fas
Fas Ligand
Fcr RIIB/C
Ferritin
FGF-4
FGF-6
FGF-6
FGF-7
FGF-9
Fit-3 Ligand
FLRG
Follistatin
Fractalkine
FSH
Furin
Galectin-7
GCP-2
G-CSF
GDF-15
GDNF
GITR
GITR Ligand
GM-CSF
GRO
GROα
GH
HB-EGF
HCC-4
hCG (intact)
HGF
HVEM
I-309
ICAM-1
ICAM-2
ICAM-3
IFNγ
IGF-1 SR
IGFBG-1
IGFBP-2
IGFBP-3
IGFBP-4
IGFBP-6
IGF-I
IGF-I SR
IGF-II
IL-1α
IL-1β
IL-1 R II
IL-1 R4/ST2
IL-1 RI
IL-1 sRI
IL-10
IL-10 Rα
IL-10 Rβ
IL-11
IL-12
IL-12 p40
IL-12 p70
IL-13
IL-13 Rα-2
IL-13 RI
IL-15
IL-16
IL-17
IL-17B
IL-17C
IL-17F
IL-17R
IL-18 BPα
IL-18 Rβ
IL-1ra
IL-2
IL-2 Rβ
IL-2 Rγ
IL-2 Ra
IL-21R
IL-22
IL-28A
IL29
IL-3
IL-31
IL-4
IL-5
IL-5 Rα
IL-6
IL-6 sR
IL-7
IL-8
IL-9
Insulin
IP-10
I-TAC
LAP
Leptin
Leptin R
LIF
LIGHT
LIMPII
L-Selectin
LH
Lymphotactin
LYVE-1
Marapsin
MCP-1
MCP-2
MCP-3
MCP-4
M-CSF
M-CSF R
MDC
MICA
MICB
MIF
MIG
MIP-1α
MIP-1β
MIP-1δ
MIP-3α
MIP-3β
MMP-1
MMP-10
MMP-13
MMP-2
MMP-3
MMP-7
MMP-8
MMP-9
MPIF-1
MSPα
NAP-2
NCAM-1
NGF R
Nidogen-1
NrCAM
NRG1-β1
NT-3
NT-4
Oncostatin M
Osteopontin
OPG
PAI-I
PARC
PDGF Rα
PDGF Rβ
PDGF-AA
PDGF-AB
PDGF-BB
PECAM-1
PIGF
PF4
Procalcitonin
Prolactin
PSA-free
PSA-total
RAGE
RANK
RANTES
Resistin
S-100b
SAA
SCF
SCF R
SDF-1
SDF-1β
SAA
sgp130
Shh N
Siglec-5
Siglec-9
ST2
sTNF RI
sTNF RII
TACE
TARC
TECK
TGFα
TGFβ1
TGFβ2
TGFβ3
TPO
Thyroglobulin
Tie-1
Tie-2
TIM-1
TIMP-1
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TIMP-4
TNFα
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TRAIL R2
TRAIL R3
TRAIL R4
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